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13. ABSTRACT *(Maximum 200*

Further progress has been made at the pre-clinical level in the study of iron deprivation as a potential treatment modality for breast cancer. The MDA-MB-231 cell line appears to be resistant to combined treatment with HES-DFO and monoclonal antibodies against the transferrin receptor; studies with the SK-BR-3 cell line are now under way. Preliminary data suggest that 231 cells may cease dividing but not undergo apoptosis when acutely deprived of iron; this is in contrast to the findings with the 38C13 lymphoma. The possibility thus arises that distinct gene activation pathways may occur in response to iron deprivation. New data indicate that combined treatment may produce supra-additive increases in serum IL-6 levels and thus offer further support for the hypothesis that enhanced macrophage activation and an augmented acute phase response play a role in the toxicity of iron deprivation. New data also indicate that monoclonal antibodies against the transferrin receptor inhibit lymphocyte activation in vivo.

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John D Kemp 2/26/98

PI - Signature Date

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Introduction

The problem being addressed is the need for improved therapy of metastatic breast cancer. There are now clear indications that iron deprivation treatment is useful clinically against several tumor types. We have previously published evidence that iron deprivation inhibits breast cancer cell lines in vitro and iron deprivation can be employed in vivo (1,2). We are continuing to analyze mechanisms underlying the anti-tumor effects of iron deprivation treatment and its potential utility in pre-clinical experimental systems involving breast cancer.

Body

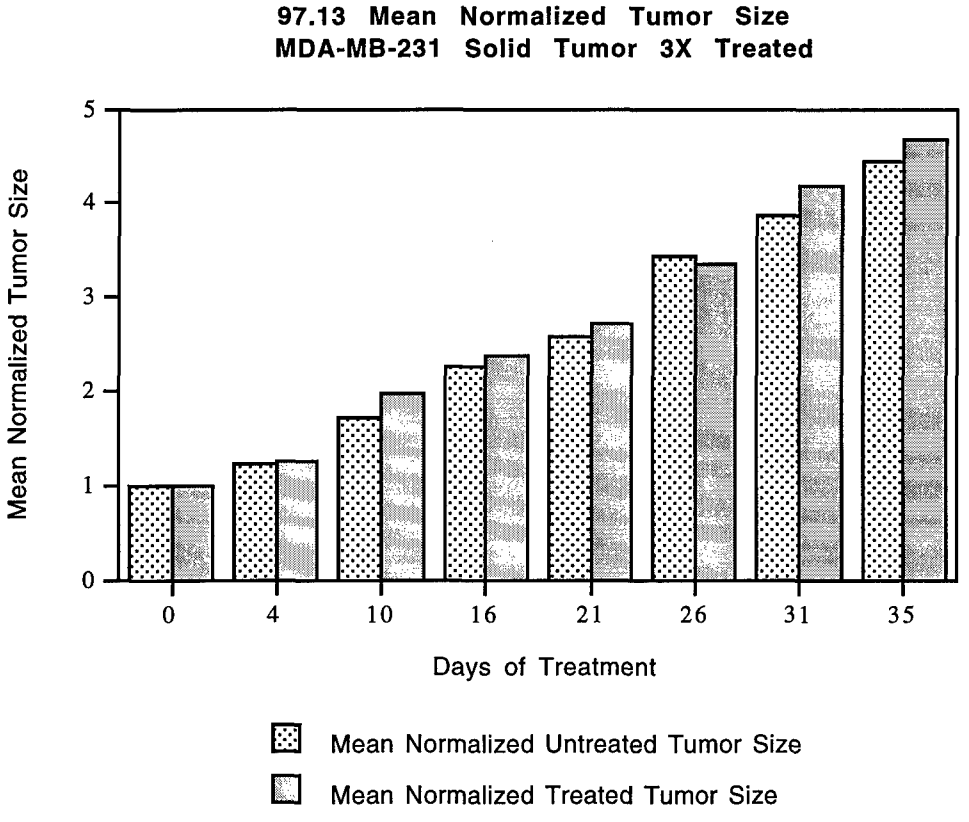
During the period from December 23, 1996 to January 22, 1998, our research efforts were primarily focused on Tasks 3 and 4, the central and most important Tasks of our application. We will therefore begin our discussion with these Tasks.

Our goal for Task 3 was to "Ascertain the growth kinetics of the SK-BR-3 and MDA-MB-231 cell lines in nude mice and treat them with HES-DFO and the IgG ATRA pair A27 and E2.3". We have now been successful in conducting two full-scale experiments with MDA-MB-231. The data from the first such experiment, 97-13, are shown below in Figure 1. This data was presented at the Army Breast Cancer Research Meeting in Washington, D.C.

Experimental Details For Figure 1

One million 231 cells were injected subcutaneously into female athymic nude mice along with 5 mg/ml of matrigel in a 0.1 ml. volume. Measurable tumors were detected in 5-6 weeks. Treated groups were then given 0.5 ml of a 26 millimolar solution of HES-DFO i.p. on a daily basis and 1.5 mg each of two monoclonal antibodies against the human transferrin receptor (A27.15 and E2.3) every three days, also i.p. The protocol is similar in design to that which was employed against the murine lymphoma 38C13 in vivo (2) but employs the same antibodies that were used against 231 in vitro (1).

Figure 1



Conclusions

Although this a only a single experiment, it would appear that established MDA-MB-231 tumors show no sensitivity to the initial treatment protocol in vivo. It should be kept in mind, however, that 231 showed the least sensitivity to combined iron deprivation treatment in vitro (1). Thus, it will be necessary to move on to a test with SK-BR-3 cells. The latter cells showed significantly greater sensitivity to combined iron deprivation treatment in vitro (1). If SK-BR-3 cells show significant sensitivity, then we will conclude that breast cancers differ in their responses to combined iron deprivation treatment and proceed to investigate the basis for the difference. If SK-BR-3 cells are also insensitive, however, then it would be necessary to entertain the notion that breast cancers as a class might be resistant. It would then be important to understand how they as a class differ physiologically from lymphomas, neuroblastomas, and transitional cell carcinomas.
(As second experiment, carried out in essentially identical manner, confirmed the results of the first. While these two experiments were carried out with MDA-MB-231 cell line, we now have the first experiment with SK-BR-3 underway).

While awaiting the results of the SK-BR-3 experiments, and in response to the unexpected insensitivity of the 231 cells, we have also elected to undertake some focused additional experiments to assess the response of MDA-MB-231 to acute specific iron deprivation in vitro. Preliminary data suggests that 231 cells, unlike the 38C13 lymphoma cells (3), fail to undergo apoptosis. They do appear to cease cell division (as would be expected at minimum in the absence of iron) but they also appear to be capable of surviving for extended periods of time. If this can be confirmed and analyzed further, it will suggest that different gene programs can be activated by iron deprivation in different tumors.

Our goal in Task 4 was to "Estimate the potential toxicity associated with iron deprivation treatment".

As was noted in the last Annual Report, we developed data which indicated that mice undergoing treatment for six to seven days with HES-DFO and two monoclonal antibodies against the transferrin receptor developed a puzzling form of systemic toxicity that was not obviously attributable to infection at autopsy. By means of EPR (electron paramagnetic resonance) we were able to gather evidence indicating that a third of the mice had elevated whole blood nitric oxide levels and that all of the mice appeared to have elevated ceruloplasmin levels. The latter is an acute phase reactant which is most likely to be induced by Interleukin 6. We have recently acquired additional data (Expt. 97.29 below) which suggests that IL6 levels are indeed elevated in the treated mice.

97.29 : IL-6 levels in Sera of C3H/HeN Mice Undergoing Treatment With Mabs, HES-DFO, or the two Combined
Data expressed as pg/ml of IL-6

	Mean	Std. Error
Control	89.25	40.647
MAbs. alone 8 Days	91.50	1.607
HES-DFO alone 8 Days	140.83	61.858
Both Combined 8 Days	191.30	81.254

Experimental Details for 97.29

The antibodies and HES-DFO were given at the same doses, with the same timing, and by the same routes as noted for Figure 1. In this case, however, rat anti-mouse transferrin receptor antibodies C2 and RL34 were used rather than mouse anti-human receptor antibodies. Mouse serum IL-6 was measured with duplicate samples with an ELISA kit from Endogen, Inc. The results are from the first experiment.

Conclusion

One tentative conclusion from the preliminary experiment is that while antibody treatment alone may not elevate IL-6 levels (no difference from control), HES-DFO may elevate them. A second tentative conclusion is that combined treatment may result in a suppra-additive increase in Il-6 production.

These studies continue to be consistent with the hypothesis that we mentioned in last year's Annual Report; i.e., that there is enhanced macrophage activation as a result of the iron deprivation produced by combined treatment. Moreover, that activation may be leading to both a significant acute phase response and to an increased propensity for the production of significant levels of nitric oxide.

We have also been able to capitalize on an opportunity to evaluate the effects of monoclonal antibody treatment alone on lymphocyte activation in vitro in the context of two types of allograft rejection experiments. These experiments were based on our own prior studies which indicated that such antibodies selectively inhibited lymphocyte activation in vitro. In particular, those studies suggested that generation of cytotoxic T lymphocytes (CTLs) and the proliferation of the TH1 subset of helper T cells (those most responsible for cell mediated immunity in graft rejection) were preferentially inhibited in vitro.

The first set of experiments have to do with the inhibition of splenic CTLs. The results from a representative experiment (one of four) are shown in Table 2.

Table 2

	P815					
EFFECTOR	3.1	6.2	12.5	25	50	100
B6 SPLEEN	-2.27	-3.56	-2.63	-2.10	-1.25	-4.12
B6 SPLEEN + P815	8.25	15.73	27.06	39.23	47.94	50.14
B6 SPLEEN + P815 +NRIgG	5.41	11.79	20.54	32.13	37.56	44.70
B6 SPLEEN + P815 +C2	-1.70	0.82	-2.34	-1.57	-3.55	-3.00
B6 SPLEEN + P815 +RL34	-2.79	-0.78	-4.53	-3.06	-3.47	-4.05
B6 SPLEEN + P815 + C2 + RL34	-2.58	-2.59	-2.72	-3.29	-3.70	-4.16
B6 SPLEEN + P815 +C2 + RL34	-3.25	-2.00	-1.67	-4.07	-1.69	-4.20

Monoclonal antibodies against the transferrin receptor (C2 and RL34) block the development of alloantigen specific CTLs in vivo.

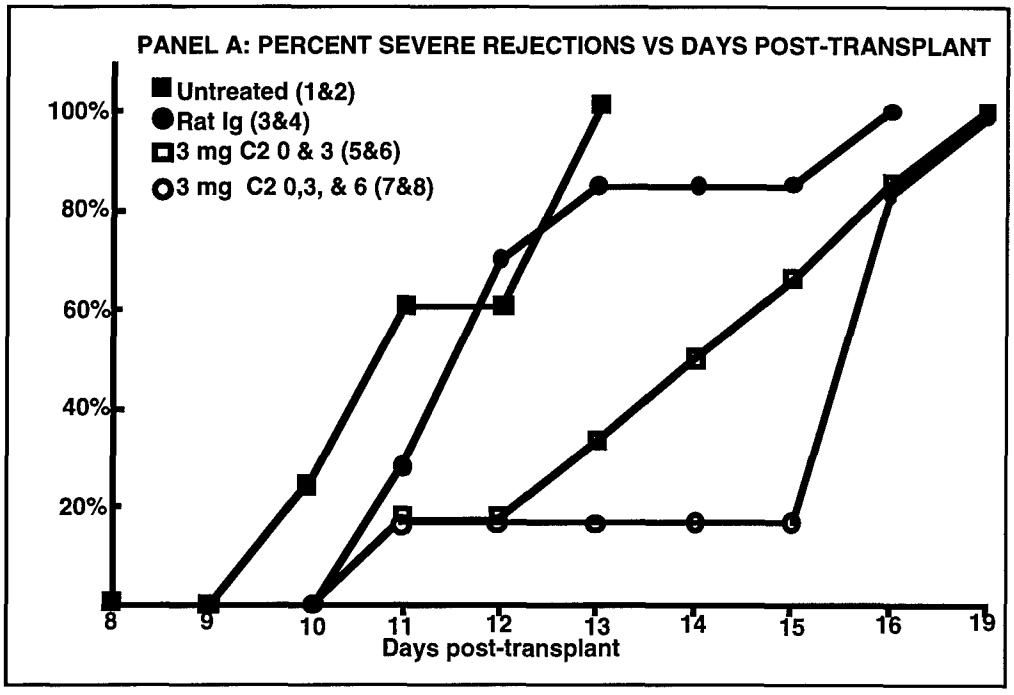
Experimental Details and Conclusion

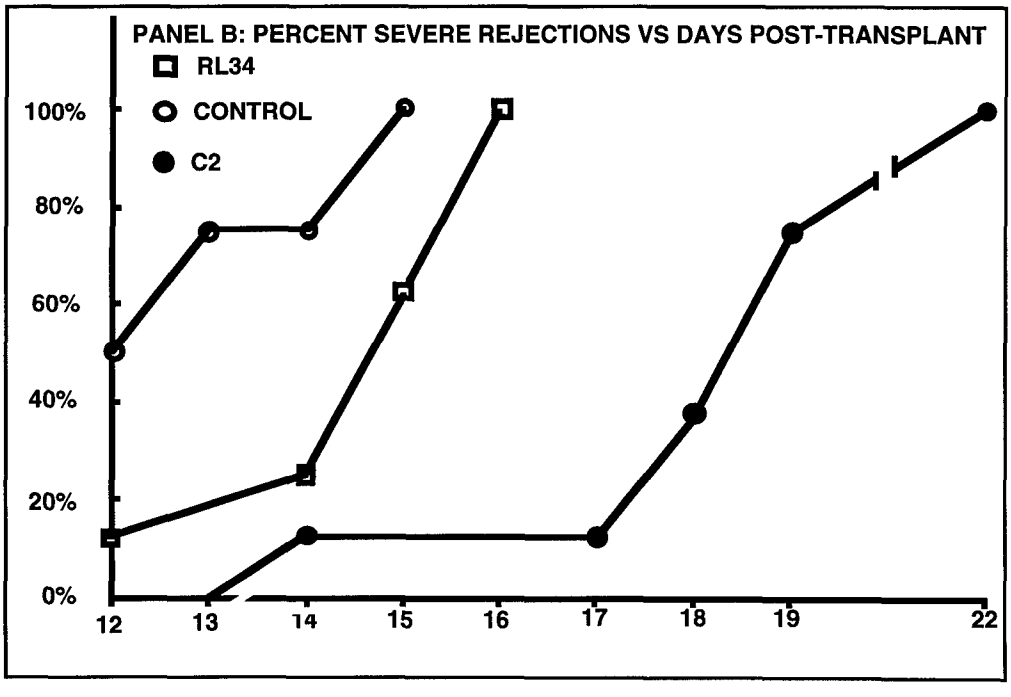
C57BL/6 mice were injected i.p. with 0.1 ml of ascites containing the P815 mastocytoma tumor and their spleens were harvested 10 days later and CTL activity was measured by chromium release assay. Treatments consisted of monoclonal antibody or control rat IgG injections on days 0, 3, and 6. Mice receiving either C2 or RL34 only received 3 mg of each antibody per injection. Mice receiving both antibodies received 1.5 mg of each antibody per injection. The central column headings reflect effector to target ratios and the data underneath are percent specific lysis. The experiment is representative of four performed.

The data are surprising in that they show an essentially complete inhibition of the generation of CTL with the doses of either antibody (alone or together) that have been employed in anti-tumor experiments in vivo. This was surprising because the antibodies alone had so little effect on the tumors. The data suggest that receptor epitopes on lymphocytes in the spleen are readily saturated in vivo and that the degree of inhibition of lymphocyte function in vivo is equal to that noted in vitro. The potential long-range significance of this observation is discussed after the next set of experimental data.

The second set of experiments have to do with the inhibition of skin graft rejection. The idea here was to more specifically inhibit the function of TH1 type helper cells in vivo. The results from two experiments (with different protocols) are shown in Figure 2.

Figure 2


PANEL A: PERCENT SEVERE REJECTIONS VS DAYS POST-TRANSPLANT
Untreated (1&2)
Rat Ig (3&4)
3 mg C2 0 & 3 (5&6)
3 mg C2 0,3, & 6 (7&8)
100%
80%
60%
40%
20%
8
9
10
11
12
13
14
15
16
19
Days post-transplant


PANEL B: PERCENT SEVERE REJECTIONS VS DAYS POST-TRANSPLANT
RL34
CONTROL
C2
100%
80%
60%
40%
20%
0%
12
13
14
15
16
17
18
19
22

Experimental Details and Conclusion

C57Bl/6 mice were challenged with syngeneic or allogeneic tail skin grafts. the latter were from fully incompatible C3H mice. Syngeneic grafts were accepted throughout the course of the experiements in all cases. The data for severe rejection events for the allogeneic grafts are presented. In the top panel, the groups are either untreated, treated with non-immune rat IgG, treated with the monoclonal antibody C2 on days 0 and 3 (open squares), or treated with C2 on days 0,3, and 6 (open circles). In the bottom panel, the groups are untreated control, treated with the monoclonal antibody RL34 (open squares), or treated with C2 (closed circles). The data in the top panel indicate that three doses of equal amounts of a rat anti-mouse antibody C2 (in the same amounts previously administered in the tumor experiments) produce a significant delay in skin rejection while two doses are a little less effective. The presence of an unequivocal difference in such in vivo experiments was once again surprising in view of the relative lack of anti-tumor effect of C2 alone. Another interesting finding is that shown in the lower panel. Surprisingly, it appears that the antibody C2 produces a significant inhibitory effect while the antibody RL34 has a much more modest, potentially insignificant effect. This is remarkable because both antibodies were equally effective in the inhibition of CTLs in vivo. We believe that the most likely explanation for this effect derives from the fact that C2 is known to be a high affinity antibody and that it may cross-link and degrade receptors on cells with lower receptor densities than does RL34. Based on our prior studies (4), there is reason to believe that CD4+ effector T cells (the most important cells in graft rejection) have lower receptor densities than CD8+ CTLs.

Taken together, both sets of experiments indicate that ATRAs have more immunosuppressive effects than we had anticipated. On the one hand, this would mean that if the antibodies were to be employed as part of anti-tumor regime then the patients would have to be considered as being immunosuppressed, particularly with respect to cell mediated immune responses. Therapeutic immunosuppression is, however, clearly of use in some situations such as acute GVH disease and it may well be that the antibodies could be employed in the treatment of this complication of bone marrow transplantation, which is itself one therapeutic tool in the treatment of breast cancer.

Conclusions

Further progress has been made in the study of iron deprivation as a potential treatment modality for breast cancer. The MDA-MB-231 cell line exhibited unexpected resistance to treatment in vivo. As originally planned, we will continue experiments with the SK-BR-3 tumor which was more sensitive in vitro. Preliminary tissue culture experiments have raised the possibility that tumors will exhibit distinctly different responses to acute iron deprivation. Further data continues to support the hypothesis that acute iron deprivation may lead to macrophage activation, an acute phase response, and elevated nitric oxide production. It remains to be seen whether or not such a thing would happen in human beings. Surprising new data indicates that monoclonal antibodies against the transferrin receptor are, in themselves, capable of causing surprising inhibition of the generation of cytotoxic T lymphocytes in vivo and prolongation of the process of skin graft rejection. This may suggest a potentially interesting treatment modality for acute graph versus host disease which a significant problem in bone marrow transplantation treatment for breast cancer.

Progress to date indicates that no insurmountable barriers have been encountered and that all of the proposed research can be accomplished within the time frame originally proposed. Finally, one paper is attached which arose from a long-standing project of a graduate student; support from this made an indirect contribution to the completion of this work. The paper describes the intron/exon structure of the human transferrin receptor gene. The knowledge acquired will facilitate future studies into the role of the transferrin receptor in the behavior of breast cancer cells. In particular, it may allow us to construct a targeting vector to knock out the gene in human breast cancer tumor cells.

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Exon/intron structure of the human transferrin receptor gene[1]

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Exon/intron structure of the human transferrin receptor gene[1]

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Abstract

A PCR-based intron jumping strategy has been utilized to investigate the exon/intron structure of the human transferrin receptor gene and determine the sequences of exon/intron junctions. There are 18 exons and introns 5′ to a large exon encoding the last translated segment and a sizable 3′ untranslated segment. All of the translated segments are encoded by exons 2–19. The tight turn motif, which is critical to the process of endocytosis, is encoded by exon 3. Based on recent studies of human/chicken receptor chimeras, it appears that the residues most likely to be involved in transferrin binding are encoded by exons 17–19. Exon 12 exhibits the greatest degree of homology with the gene for the prostate specific membrane antigen. A polymorphism has been tentatively identified at nucleotide position 519 in exon 4; the presence of either adenine or guanine should result in either serine or glycine, respectively, at position 142 of the amino acid sequence. This analysis of genomic structure will permit further detailed studies of the regulation, expression and evolution of the human transferrin receptor gene. © 1997 Elsevier Science B.V.

Keywords: Iron; PCR; Polymorphism; Prostate-specific membrane antigen

1. Introduction

In vertebrates, the dominant mechanism for cellular iron uptake involves a process in which transferrin (the principal iron transport protein in plasma) is bound by a homodimeric transmembrane glycoprotein, the transferrin receptor (Aisen and Listowsky, 1980). While cDNA sequences for the human transferrin receptor (HuTfR) have been available for some time (Schneider et al., 1984; McClelland et al., 1984), the details of its genomic structure have remained undefined. The best available estimate for exon/intron organization is that of McClelland and colleagues (McClelland et al., 1984). They conducted an electron microscopic study of cDNA/genomic DNA heteroduplexes and concluded that there were 19 exons and 18 introns. They also made estimates of the sizes of each exon and intron. We utilized their estimates to initiate a PCR-based 'intron-jumping' strategy for making exact determinations of exon sizes and exon/intron boundaries.

2. Materials and methods

2.1. Primer design

Using the cDNA sequence and estimates of mean exon sizes determined by McClelland and co-workers (McClelland et al., 1984), hypothetical exon boundaries were assigned. Realizing that such assignments would contain errors, primers were designed for sequences which were thought to reside in, or near, the centers of the putative exons. Primers for these sequences were evaluated with respect to primability and stability using the Amplify computer software (William Engels, Department of Genetics, University of Wisconsin, Madison, WI, USA). Primability was set at 60% and stability was set at 30%. The primers were shortened or lengthened (final lengths are between 20 and 32 nucleotides) until the greatest specificity was achieved.

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[1] This work was presented in part in abstract form at the 1996 Experimental Biology Meetings in New Orleans, LA, USA.

Abbreviations: PCR, polymerase chain reaction; HuTfR, human transferrin receptor; PSM, prostate-specific membrane antigen; HSP, high-scoring segment pair; cDNA, DNA complementary to RNA; bp, base pair(s).

PII S0378-1119(97)00356-9

The final evaluation step was completed using the Oligo 4.0 computer software (National Biosciences, Plymouth, MN, USA). This program was used to check for possible hairpin loops and primer dimers. Primers were accepted if the hairpin loops had a free energy value greater than $-1.0\Delta G$. After a primer fulfilled the above criteria, it was synthesized using the phosphoramidite method on an Applied Biosystems 392 DNA/RNA synthesizer (Perkin Elmer, Foster City, CA, USA) and purified using KwikSep® Excellulose® Plastic Desalting Columns with a gel exclusion limit of 5000 MW (Pierce, Rockford, IL, USA).

The introns of the HuTfR were amplified and the exon/intron boundaries were determined by sequencing PCR products generated with the following primers.

Intron 1:
5′-cctcagagcgtcgggatatcgggtggcggc (EX1UP);
5′-gaatgctgatctagcttgatccatcat. (EX2DN)
Intron 2:
5′-cagttcagaatgatggatcaagctagatca (EX2UP);
5′-agtcccatagcagatacttccacta (EX3DN).
Intron 3:
5′-gtgtagtggaagtatctgctatgggac (EX3UP);
5′-ctccgacaactttctcttcaggtc (EX4DN).
Intron 4:
5′-tgacctgaagagaaagttgtcggag (EX4UP);
5′-atgttgatcacgccagactttgct (EX5DN).
Intron 5:
5′-gaatttaaactcagcaaagtctggcgtgat (EX5UP);
5′-cttactatacgccacataacccccaggatt (EX6DN).
Intron 6:
5′-aatcctgggggttatgtggcgtatagtaag (EX6UP);
5′-gtaccaaaattagcatggaccagtttac (EX7DN).
Intron 7:
5′-ggtaaactggtccatgctaattttg (EX7UP);
5′-gtctggtccatgtatatcaacacaccaat (EX8DN).
Intron 8:
5′-aattggtgtgttgatatacatggaccagac (EX8UP);
5′-gctctggagattgtctggacaggtatatt (EX9DN).
Intron 9:
5′-tatacctgtccagacaatctccagagctg (EX9UP);
5′-gtgagcttcacattcttgctttctgagg (EX10DN).
Intron 10:
5′-agaaagcaagaatgtgaagctcactgt (EX10UP);
5′-acatctgggcaagtttcaataggag (EX11DN).
Intron 11:
5′-ctcctattgaaacttgcccagatgt (EX11UP);
5′-actccaactggcaaagataatgcttctgct (EX12DN).
Intron 12:
5′-ccagcagaagcattatctttgccagttgga (EX12UP);
5′-caagaaccgctttatccagatta (EX13DN).
Intron 13:
5′-ttcgtccctgcatttaaaggctttcactta (EX13UP);
5′-caacagtgggctggcagaaaccttgaagttgc (EX14DN).
Intron 14:
5′-gtttctgccagcccactgttgtatacgct (EX14UP);
5′-tgctggcccagttgctgtcctgatatagaa (EX15DN).
Intron 15:
5′-ctgggcaatttctatatcaggacagcaact (EX15UP);
5′-gaatatgcaaggaaagggaaagcagcattg (EX16DN).
Intron 16:
5′-gaatcccagcagtttctttctgtttttgc (EX16UP);
5′-aaagcagttggctgttgtacctctcatag (EX17DN).
Intron 17:
5′-ggactatgagaggtacaacagccaactgc (EX17UP);
5′-cactctcatgacacgatcattgagtttctt (EX18DN)
(three nucleotides of the EX18DN primer are in intron 18).
Intron 18:
5′cttccagactaacaacagatttcgg (EX18UP);
5′-ctagagccaactggtttctgaacagcgtt (EX19DN).

2.2. PCR conditions

Due to the size of some of the introns of the HuTfR (150–4800 bp), the Expand™ Long Template PCR System (Boehringer-Mannheim, Indianapolis, IN, USA) was used. The PCR reactions were carried out following the Expand kit specifications with slight modifications. After the first 10 cycles, the elongation time was increased by 20 s after every third cycle until a total of 30 cycles were completed. For all intron amplifications (except intron 1) an initial elongation time of 2 min was utilized. Owing to the size of intron 1 (approximately 4.8 kb), the initial elongation time was increased to 3 min. The PCRs were carried out using either a Hybaid Omnigene or Perkin Elmer 9600 thermal cycler. The primary source of the genomic DNA template utilized for PCR reactions was a single preparation of DNA obtained from a single human placenta. Additional PCR reactions with plasmid DNA templates were also performed as discussed in Section 2.3.

2.3. Sequence determinations

The PCR products were examined on 1.5% (w/v) ethidium bromide stained agarose gels (Gibco BRL-ultraPURE Agarose, electrophoresis grade, Gaithersburg, MD, USA). The electrophoresis was carried out at 75 W. The desired PCR products were excised from the gel and the DNA was eluted using a gel extraction kit (Qiagen, Chatsworth, CA, USA). Following the purification of the extracted DNA, it was sequenced using the PRISM® Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA, USA) and the ABI PRISM® 373A sequencer with version 2.0.15 software (Perkin Elmer). Sequencing was performed by the DNA Core Facility at The University of Iowa.

Two plasmids containing genomic fragments of the

HuTfR gene were also used in some sequencing reactions. These plasmids, known as pTR76BB 13.0 and pTR17BS 12.8, were generous gifts from Dr. Lukas Kühn (ISREC Genetics Unit, Epalinges, Switzerland). These plasmids contained inserts obtained from the recombinant phage clones λ76 and λ17 which were originally isolated from the mouse L cell line 9A4 which had been transformed with DNA from the human T-cell lymphoma line, MOLT-4 (Kühn et al., 1984). The insert in pTR76BB 13.0 is a *Bam*HI–*Bam*HI fragment from λ76, which is centrally located in the gene and which was thought to contain exons 2–11 (McClelland et al., 1984). The insert in pTR17BS 12.8 is a *Bam*HI–*Sal*I fragment from λ17 which is located 3′ in the gene and which was thought to contain exons 12–19 (McClelland et al., 1984). The inserts were subcloned into the pAT153 plasmid, a derivative of pBR322 (L. Kühn, personal communication). In the situations which required sequencing of these plasmids, the reactions were carried out by using 0.5 μg of the appropriate plasmid in conjunction with the appropriate primer. Sequencing of the plasmids was also performed by the DNA Core Facility at The University of Iowa using the same automated sequencing system as described above.

2.4. Determining exon/intron boundaries

The exon/intron boundaries were determined by identifying the points at which the sequence of the PCR products diverged from that of the available cDNA sequences (Schneider et al., 1984; McClelland et al., 1984). The exon/intron splice sites were further verified by the presence of the intron splice site consensus sequences; i.e., the first two and last two nucleotides of introns adhered to the GT/AG rule (Breatnach et al., 1978).

2.5. Analysis of a sequence difference in exon 4 via restriction enzyme analysis of a PCR product

The primers used for the evaluation of a possible HuTfR polymorphism in exon 4 were 5′-tgacctgaagagaaagttgtcggag (EX4UP) and 5′-gttctacctttttcccctaccagtatagttt (INT4DN4). These primers were used both for PCR and sequencing reactions. The restriction enzyme maps of the PCR product were analyzed using DNASIS-Mac V2.0 software (Hitachi). The exon 4 PCR products were digested with *Ban*I at 50°C. The PCR reactions and gel analyses were performed as described above.

2.6. Computer-assisted analysis of sequence homology

Sequence homology between the genes for the HuTfR and the human prostate specific membrane antigen (PSM) was assessed with the Basic Local

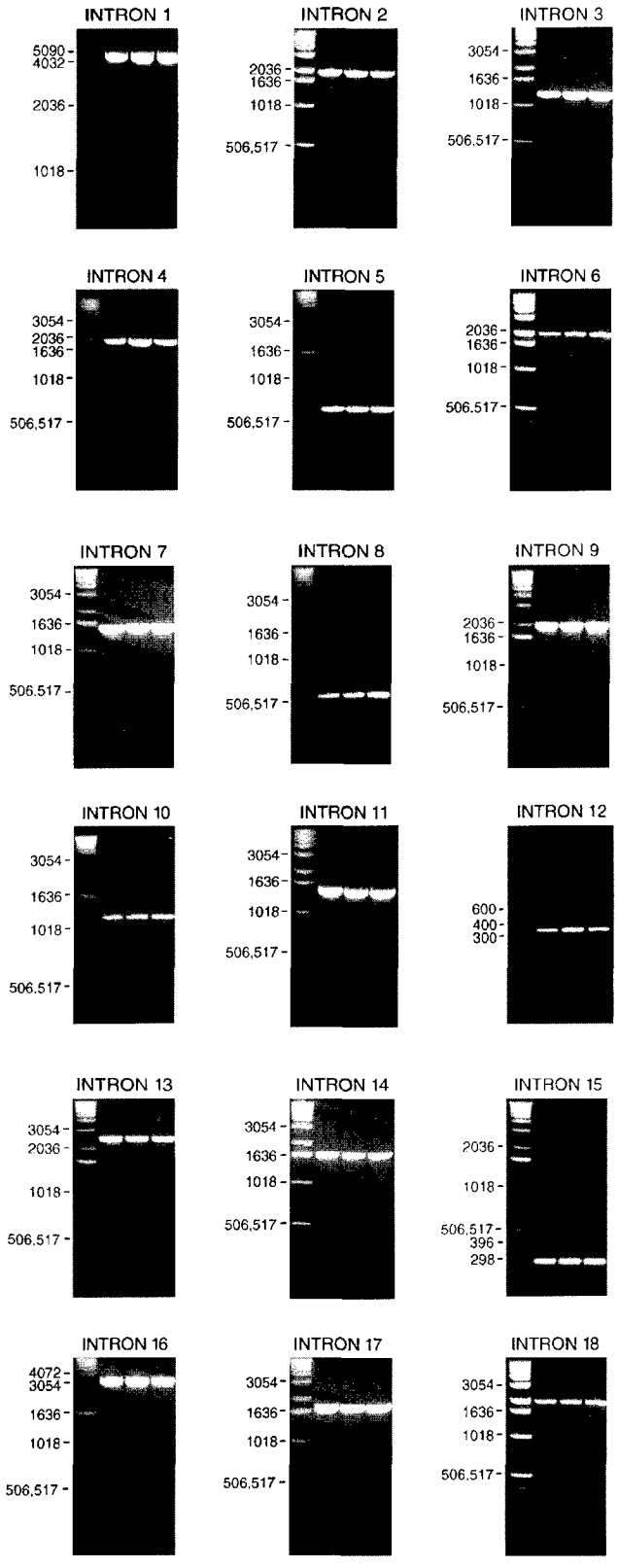

Fig. 1. Agarose gel electrophoresis of PCR products for intron 1-18. PCR primers and procedures, as well as gel procedures, are described in Materials and Methods.

Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/index.html). The non-redundant Genbank database was queried with the nucleotide sequence of the HuTfR using the advanced blastx program with the standard default settings (Matrix = BLOSUM62).

3. Results

3.1. Initial jumping of proximal and distal introns by PCR

The estimates of exon sizes provided by McClelland and colleagues (McClelland et al., 1984) were employed to assign approximate exon/intron boundaries and select sequences for PCR primers. The sequences selected were thought to be in, or near, the centers of exons. This strategy allowed us to produce PCR products that were of the expected size for crossing proximal introns 1–5, as well as distal introns 17 and 18 (Fig. 1). However, the initial attempts at jumping the more centrally located introns 6–10 and 16 failed. We hypothesized that the sequential assignment of exon boundaries based on the estimates of mean exon sizes had resulted in an accumulation of error and that some of the PCR reactions had failed because the primers corresponded to sequences that were not confined to a single exon. A further indication that our initial boundary estimates were partly erroneous was that the first attempt to jump intron 10 yielded a smaller than expected PCR product of approximately 100 base pairs. The latter product (not shown) was consistent with the amplification of a portion of a single exon, most likely either 10 or 11, without jumping an intron.

3.2. Realignment of boundary estimates by partial sequencing of plasmids containing genomic DNA for the TfR

In order to expedite the process of realignment, we elected to sequence small portions of plasmids known to contain segments of the transferrin receptor genomic sequence (see Materials and Methods). Based on prior work (McClelland et al., 1984), we proceeded on the assumption that exons 6, 10 and 11 would be contained within the insert of pTR76BB and exon 17 would be contained within the insert of pTR17BS. Thus, sequencing was initiated using the upstream primer from exon 6 with pTR76BB and the downstream primer from exon 17 with pTR17BS. In addition, both primers which had been employed in the attempt to jump intron 10 were utilized to initiate sequencing with pTR76BB. Sequencing reactions were successfully performed in all three cases and this allowed us to define precisely the 3′ border of exon 6, the 5′ and 3′ borders of what turned out to be exon 10, and the 5′ border of exon 17 (Table 1).

3.3. Final jumping of all introns by PCR

With the sequencing information obtained from the plasmids, we were able to realign the cDNA sequence and once again employ the estimates of mean exon size to design additional primers (see Materials and Methods). These new primers allowed us to jump all of the remaining introns by PCR (Fig. 1). Partial sequencing of the PCR products then allowed us to define exon/intron boundaries (Table 1) and exon sizes (Table 2). The 5′ and 3′ boundaries of exon 1 are not stated because they appear to vary (Owen and Kühn, 1987).

3.4. Comparison of exon sequences to previous Genbank entries for the human transferrin receptor

When we compared the sequences we obtained from the placental PCR products to the human cDNA sequences on file in Genbank (accession numbers M11507 and X01060), we observed a difference at position 519 (exon 4), based on previous position assignments (McClelland et al., 1984). While they as well as Schneider and colleagues (Schneider et al., 1984) reported an adenine at that position, we detected a guanine. A restriction map analysis indicated that the presence of a guanine would create a new *Ban*I restriction site which would in turn result in the production of two fragments (200 and 50 bp) when a 250 bp PCR product containing position 519 was partially digested (see Materials and Methods). As can be seen in Fig. 2, the expected fragments were observed when the PCR product from placental DNA was partially digested with *Ban*I. However, no such fragments were seen when the PCR product from the pTR76BB plasmid was treated in the same manner. The results suggested that the pTR76BB plasmid had an adenine at position 519 and this was confirmed by sequencing (see Materials and Methods). A change from adenine to guanine at position 519 should result in the substitution of a glycine for the previously reported serine at position 142 of the amino acid sequence.

We also detected two other nucleotide sequence differences when comparing our sequence with those already available. Of these, the first was in exon 7 at position 884, once again based on previous position assignments (McClelland et al., 1984). While they reported a guanine at that position, we detected a cytosine. Schneider and colleagues also reported a cytosine at position 884 (Schneider et al., 1984). In either case, the amino acid sequence is the same. The second difference was in exon 19, 86 nucleotides downstream

Table 1
Sequences at exon/intron boundaries in the human transferrin receptor gene

Intron number	Boundary sequences (5′–3′)[a]
1	variable[b] .... ATTCTGATACCTGAGgttcttctg
2	tctaacttgGTAAGA .... TTTTCCGCAACACAGtttggtgga
3	tcttgattgGTGAGA .... ACTTTGTCTCTTTAGgatttatga
4	caccatcaaGTGAGT .... CATTTTCTCCTCCAGgctgctgaa
5	caaagacagGTATGT .... TAATCTTTTTAACAGcgctcaaaa
6	acagttactGTAAGT .... CTAATTCTTGTCTAGggtaaactg
7	gcagaaaagGTGAGT .... TATGTTTTCTTTTAGgttgcaaat
8	tttggacatGTGAGT .... CTTTTGTTTTCCTAGgctcatctg
9	gctgtttggGTAAGT .... GCTTTCTGATTCTAGgaatatgga
10	tagaaccagGTAAAG .... TTTTCTTTTTTAAAGatcactatg
11	tcttaaaagGTAGAG .... TTTTTTCTCTTTCAGatgggtttc
12	tggctagagGTATTC .... TCCCTTCTCTTCTAGggatacctt
13	cggttcttgGTAAGT .... ACAATTTATTTTCAGgtaccagca
14	atgcaaaatGTGAGT .... TTTCTTATCTTACAGgtgaagcat
15	cagcaaagtGTAAGT .... TCTTTGTCTTTGCAGtgagaaact
16	ttttgcgagGTAAGT .... CGCATTGTCTCACAGgacacagat
17	gacataaagGTGAGC .... TAATGTCTGTTTCAGgaaatgggc
18	gtcatgagaGTAAGT .... GTGTTGTTTTCGTAGgtggagtat

[a]Exon sequences are shown in lower case and intron sequences are in upper case.
[b]See text (section 4).

Table 2
Sizes of exons in the human transferrin receptor gene

Exon	Determined size (bp)	Predicted size (bp)[b]
1	[a]	81±11
2	59	107±21
3	202	172±15
4	195	182±23
5	150	144±15
6	103	148±15
7	114	97±17
8	99	108±18
9	140	148±21
10	158	158±20
11	120	122±25
12	86	77±13
13	64	72±13
14	68	87±17
15	59	67±11
16	82	72±11
17	222	198±11
18	141	179±21
19	[a]	665±27

[a]The lengths of exons 1 and 19 are not stated because the 5′ and 3′ boundaries of exon 1 appear to be variable and the 3′ boundary of exon 19 has not yet been established.
[b]Predicted by McClelland and colleagues (McClelland et al., 1984).

from the end of the translated sequence in the 3′ *UTR*. We detected a guanine at this position, as did Schneider et al., while McClelland et al. detected a cytosine. Both of these differences may represent additional polymorphisms but we have not pursued them.

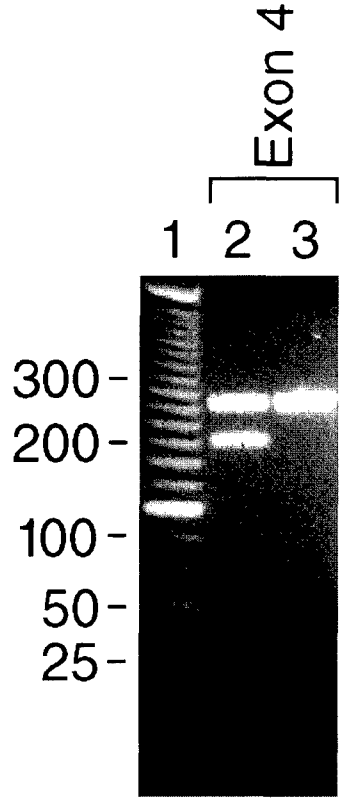

Fig. 2. The restriction enzyme partial digests of the exon 4/intron 4 PCR products. Lane 1, 25 bp standard; lane 2, exon 4/intron 4 PCR product (placental DNA template) digested with *Ban*I; lane 3, exon 4/intron 4 PCR product (plasmid pTR76BB DNA template) digested with *Ban*I.

3.5. Comparisons of the nucleotide sequences of the human transferrin receptor and the human prostate specific membrane antigen

The transferrin receptor is not known to be a member of a well-defined gene family. The only other molecule which has previously been shown to exhibit some homology with it is PSM antigen, a 100 kDa type II transmembrane glycoprotein that can function as an *N*-acetylated α-linked acidic dipeptidase (NAALADase) (Carter et al., 1996). Israeli and colleagues reported that nucleotides 1250 to 1700 of the PSM sequence exhibited 54% homology with the transferrin receptor (Israeli et al., 1993).

We studied this homology further by performing searches of the Genbank database using blastx, as detailed in Materials and Methods. A search employing the entire nucleotide sequence of the human transferrin receptor produced 11 High Scoring Segment Pairs (HSPs) with PSM. The overall sum statistic indicated the likelihood that the 11 alignments had occurred by chance was 1×10^{-59}. A general summary of the sequence regions encompassed by the various HSPs is presented in Table 3. The homology noted by Israeli and colleagues (Israeli et al., 1993) was defined by the two HSPs with the highest scores (items G and H in Table 3). The remaining HSPs were small, widely scattered runs of amino acid sequence which exhibited no clear correlation with exon delimited regions insofar as the transferrin receptor was concerned. Indeed, eight of the eleven HSPs involved more than one exon. Upon inspection, however, it appeared that the region with the highest single concentration of identical or similar residues corresponded to exon 12 of the HuTfR. This prompted us to perform additional blastx searches utilizing the individual nucleotide sequences of each translated exon of the HuTfR. This analysis showed that exon 12 was the only exon whose probability of chance alignment with PSM was low enough (2.7×10^{-6}) to be within the range defined by the nucleotide sequences of transferrin genes from other species. The HSP produced by the exon 12 specific blastx search is shown in Fig. 3.

Table 3
Homologous segments of the transferrin receptor and prostate specific membrane antigen genes[a]

HSP	Scores	PSM residues	TfR residues	TfR exons
A	40	32–41	76–85	3/4
B	64	55–108	128–181	4/5
C	41	161–185	219–243	6/7
D	78	191–234	245–288	7/8
E	53	263–274	304–315	9
F	64	288–316	328–356	9/10
G	114	339–405	370–436	10/11
H	173	407–487	439–519	11–15
I	49	549–600	563–614	17
J	100	621–697	631–707	17–19
K	42	729–747	732–750	19

[a]The homology analysis was conducted as described in Materials and Methods. HSPs are arranged inan N-terminal to C-terminal order.

4. Discussion

McClelland and colleagues estimated that there would be 19 exons and 18 introns in the HuTfR gene (McClelland et al., 1984). Our findings are entirely consistent with that estimate. The only remaining ambiguity derives from the fact that while a single exon (19) is thought to encode the entire 3 *UTR*, this has yet to be confirmed at the genomic level. Our sequencing studies do prove, however, that all of the translated segments are encoded by exons 2–19, as predicted (McClelland et al., 1984). Moreover, the estimates that McClelland et al. provided for the sizes of individual exon were generally very close to the actual values. For exons 2–18, whose size has been determined unambiguously, the McClelland estimates were within one standard deviation (SD) of the estimated size in 11 cases (exons 4, 5, 7–13, 15 and 16), two SD in three cases (exons 3, 14 and 18), and three SD in two cases (exons 2, 6 and 17). The present study therefore supports the relative accuracy of carefully performed heteroduplex analysis.

Size assignments for two exons remain ambiguous. The size of exon 1 is uncertain because the 5′ and 3′ boundaries may depend upon the promoter elements and splice sites that are used in a given cell type (Owen and Kühn, 1987). The size of exon 19 is also uncertain. We have unambiguously defined the 5′ boundary of exon 19 and determined that there are 240 bp between it and the stop codon. Furthermore, although we have confirmed the sequence of 396 bp of 3′ untranslated sequence downstream of the stop codon, we have not sequenced the remainder of that region. Since the largest available cDNA sequence does not extend to the end of the mRNA (Schneider et al., 1984), it is currently not possible to state the number of nucleotides that are expected to reside in exon 19, assuming that it does indeed encode the remainder of the mRNA. That number should be slightly larger than 2706 nucleotides, however, based on the fact that there are 240 translated nucleotides (this study) and based on the estimate that the 3′ non-coding region is slightly larger than 2466 nucleotides (Schneider et al., 1984).

It is of interest to see how the amino acids which determine major structural features of the HuTfR protein are distributed among the exons of the gene. The interest arises in part because of the controversy as to whether exons might act as minigenes for functional domains of proteins (Gilbert and Glynias, 1993;

```
TfR: 441 GFQPSRSIIFASWSAGDFGSVGATEWLE 568
         G++P R+I+FASW A +FG +G+TEW E
PSM: 409 GWRPRRTILFASWDAEEFGLLGSTEWAE 436
```

Fig. 3. Amino acid sequence alignment for residues encoded by exon 12 of the HuTfR and residues of the human PSM antigen. Glycine residue 441 is encoded by the first intact codon of the exon and is two nucleotides away from the 5′ splice site. Glutamic acid residue 568 is encoded by the last three nucleotides of exon 12. The line between the two sequences indicates those residues which are identical (same single letter code), as well as those which are non-identical but which nevertheless have a positive alignment score as determined by the scoring matrix BLOSUM62 (+).

Stoltzfus et al., 1994). A diagram depicting key relationships is shown in Fig. 4.

To begin with, exon 1 and a portion of exon 2 are untranslated. Exon 2 contains the start codon and the nucleotides encoding the first 12 (N-terminal) amino acid residues of the receptor. Exon 3 begins with the codon for the phenylalanine at position 13. This residue appears to play some role in promoting the efficiency of endocytosis (McGraw et al., 1991). Exon 3 also encodes (a) four amino acids (beginning with tyrosine at position 20 and continuing on with threonine, arginine, and phenylalanine at positions 21 through 23, respectively) that constitute the 'tight turn' structural motif which is essential for endocytosis (Collawn et al., 1990); (b) the serine at position 24 which is uniquely phosphorylated in response to phorbol ester treatment (Davis et al., 1986); and (c) the cysteine residues at positions 62 and 67 which serve as sites for covalent attachment of palmitic acid (Alvarez et al., 1990). Exon 3 ends in the midst of the codon for residue 80 (glycine) which is a little more than halfway through the transmembrane segment, according to the model recently proposed by Alvarez and colleagues (Alvarez et al., 1990).

The structure of exon 3 does not appear to provide any obvious support for the exon/minigene hypothesis (Gilbert and Glynias, 1993; Stoltzfus et al., 1994). It seems, first of all, to be too complex functionally. The residues that create the discrete tight turn motif are encoded by the same exon that encodes residues which function to anchor and insert the receptor protein in the membrane. In addition, when one compares the transferrin receptor gene to other genes with well-defined tight turn motifs, it becomes apparent that the location of the motif does not have a consistent relationship to exon/intron boundaries. Thus, while exon 3 of the HuTfR gene encodes seven amino acids which are N-terminal to the tight turn sequence (this study), exon 17 of the low density lipoprotein receptor gene encodes 26 such amino acids (Sudhof et al., 1985). Even more striking, however, is that the fact that the motif is split between exons 47 and 48 in the gene for the mannose-6-phosphate receptor (Szebenyi and Rotwein, 1994).

Exon 4 begins by completing the codon for the glycine at position 80 (an intramembranous residue) and terminates in the middle of the codon for residue 145. Exon 4 encodes (a) the extracellular cysteines at positions 89 and 98 which form critical disulfide bonds in the receptor homodimer (Jing and Trowbridge, 1987); (b) the arginine and leucine at positions 100 and 101 between which a proteolytic cleavage event produces the soluble transferrin receptor (Shih et al., 1990); (c) the threonine at position 104 which forms a site for O-linked oligosaccharide attachment and which appears to modulate cleavage at position 100 (Rutledge et al., 1994); and (d) the Lys–Arg–Lys residues at positions 128–130 which form a potential trypsin cleavage site (McClelland et al., 1984). Exon 4, like exon 3, appears to be functionally complex, and thus also does not appear to offer any obvious support for the exon/minigene hypothesis.

The residues encoded by exons 5, 6, 8 and 10–18 are not currently thought to undergo any post-translational modifications. There are, however, three sites for N-linked glycosylation in the extracellular domain (Schneider et al., 1984; Omary and Trowbridge, 1981). One of the sites (Asn–Gly–Ser; positions 251–253), which does not appear to be critical for receptor function (Williams and Enns, 1993), is encoded by exon 7. The second site (Asn–Gly–Thr; positions 317–319), which also does not appear to be critical for receptor function (Williams and Enns, 1993), is encoded by exon 9. The third site (Asn–Gly–Thr; positions 727–729), which is important for proper receptor processing and cell surface expression (Williams and Enns, 1993), is encoded by exon 19.

Based on a recent study of human/chicken transferrin receptor chimeras, it would appear that the binding sites for two mouse anti-human receptor antibodies are formed by residues within the region defined by amino acids 151–410 (Buchegger et al., 1996). It is now clear that this region is encoded by exons 5–11. The same receptor chimera studies also indicated that the transferrin binding site is located within the carboxy terminal 192 amino acids (residues 569–760) and possibly within the last 72 amino acids (residues 689–760). Residues 569–760 are encoded by exons 17, 18 and 19 and residues 689–760 are encoded exclusively by exon 19.

Our studies further confirm that the HuTfR and the PSM antigen exhibit a degree of sequence homology. There are small scattered portions of homologous amino acid sequences distributed throughout the molecules but the highest degree of homology is associated with the residues which are encoded by exon 12 of the HuTfR. The physiological significance of this at the level of protein function is not yet apparent, since the residues

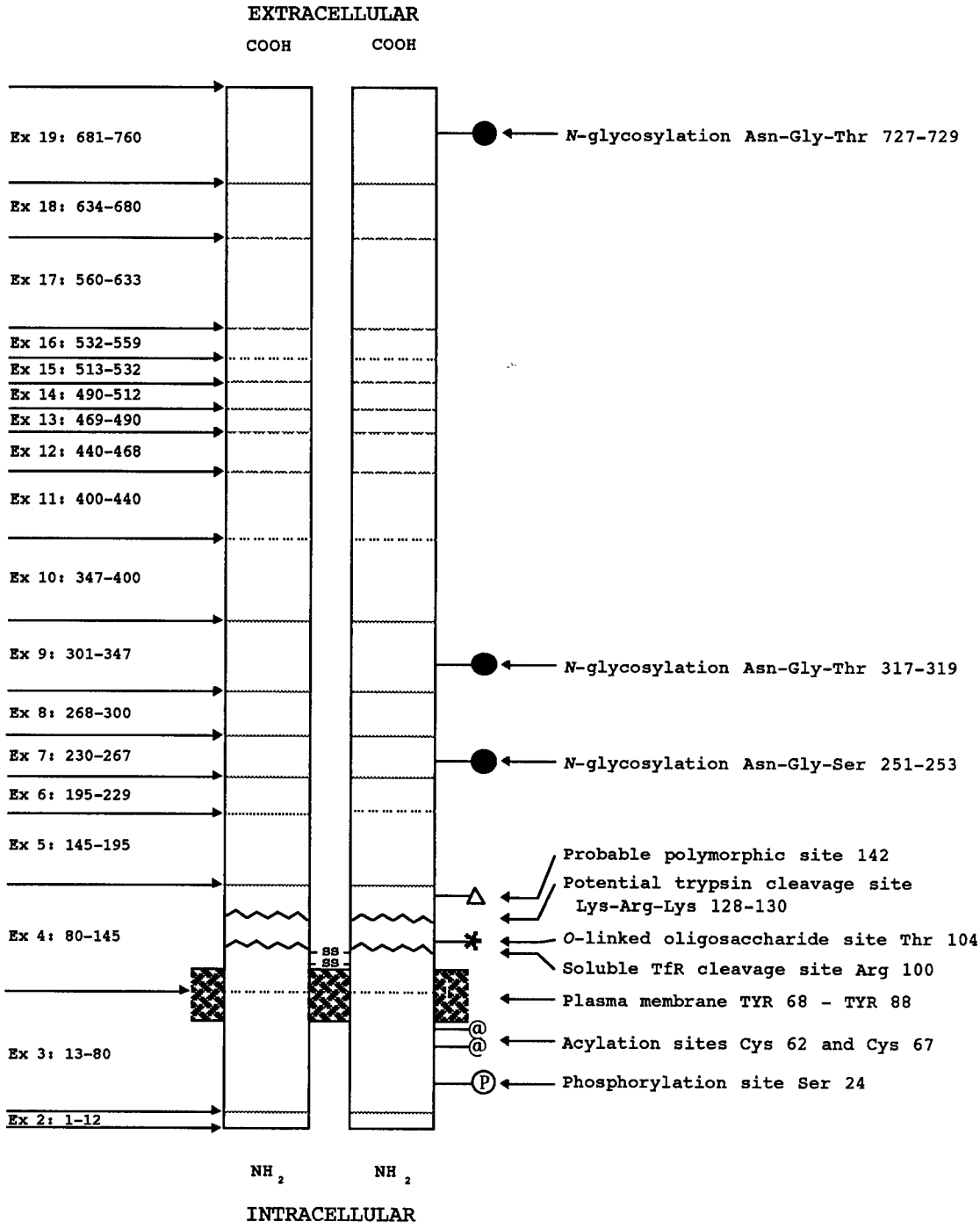

Fig. 4. Exons and structural features of the transferrin receptor.

of interest have not been assigned specific roles in either protein. In particular, the residues encoded by exon 12 are not thought to play a critical role in the formation of the transferrin binding site. As has been noted, exons 17–19, and perhaps 19 in particular, appear to be the most critical (Buchegger et al., 1996). The homologies noted for the latter exons might take on much greater significance if it turned out to be the case that the PSM molecule also binds transferrin.

From an evolutionary perspective, we interpret the finding of a larger homology in conjunction with multiple scattered smaller homologies to indicate that it was somehow functionally advantageous for the two proteins to maintain at least one common functional element as they slowly diverged. We think it less likely that the homology arose as a result of a shuffled minigene insert. In this regard, it would be interesting to compare the exon/intron structure of the PSM gene. We suspect

that several junctions will be the same. If that turns out to be the case, then the argument for considering the transferrin receptor and the PSM antigen to be members of an evolutionarily related gene family would clearly be strengthened.

Finally, we have identified an apparent amino acid sequence polymorphism in the HuTfR. The data suggest that some individuals have an adenine at position 519 in exon 4 while others have a guanine, and preliminary studies with DNA from several individuals (which will be submitted separately) indicate that this is indeed the case. Individuals with an adenine at position 519 of the nucleotide sequence should have a serine in position 142 of the amino acid sequence, while individuals with a guanine at position 519 should have a glycine. Residue 142 has not yet been implicated in any critical aspect of HuTfR function, however, and it is therefore not clear whether this polymorphism will have any significant physiological impact. It seems unlikely that the polymorphism would affect transferrin binding since residue 142 is far removed from the residues in exons 17, 18 and 19, which have been most clearly implicated in that function (Buchegger et al., 1996). It also seems unlikely that the polymorphism would affect the generation of the circulating form of the receptor since the critical cleavage site between residues 100 and 101 (Shih et al., 1990) is not very close to residue 142. However, it is certainly feasible to investigate both possibilities and studies along these lines are currently being planned.

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